Effects of the correction of particle microbial contamination and particle transit model in the rumen on *in situ* protein evaluation of grass hays

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Abstract. Effects of considering the particle comminution rate (k_c) in addition to particle rumen outflow (k_p) and the ruminal microbial contamination on estimates of by-pass and intestinal digestibility of DM, organic matter and crude protein were examined in perennial ryegrass and oat hays. By-pass k_c-k_p-based values of amino acids were also determined. This study was performed using particle transit, *in situ* and ^{15}N techniques on three rumen and duodenum-cannulated wethers. The above estimates were determined using composite samples from rumen-incubated residues representative of feed by-pass. Considering the comminution rate, k_c, modified the contribution of the incubated residues to these samples in both hays and revealed a higher microbial contamination, consistently in oat hay and only as a tendency for crude protein in ryegrass hay. Not considering k_c or rumen microbial contamination overvalued by-pass and intestinal digestibility in both hays. Therefore, non-microbial-corrected k_p-based values of intestinal digested crude protein were overestimated as compared with corrected and k_c-k_p-based values in ryegrass hay (17.4 vs 4.40%) and in oat hay (5.73 vs 0.19%). Both factors should be considered to obtain accurate *in situ* estimates in grasses, as the protein value of grasses is very conditioned by the microbial synthesis derived from their ruminal fermentation. Consistent overvaluations of amino acid by-pass due to not correcting microbial contamination were detected in both hays, with large variable errors among amino acids. A similar degradation pattern of amino acids was recorded in both hays. Cysteine, methionine, leucine and valine were the most degradation-resistant amino acids.

Introduction

Modern systems of protein rationing for ruminants are based on estimates of both protein degradability and intestinal digestibility of undegraded feed protein. However, current methodology of *in situ*-based systems may not be so well adapted to the complex digestive physiology of ruminant forestomachs and so, not accurate enough to estimate protein degradability and intestinal digestibility properly. Actually, these techniques currently present two main limitations stemming from: (i) the contamination of rumen-incubated residues with adherent microorganisms and (ii) the subevaluation of rumen residence time by not taking into account the rate of comminution and mixing of particles (k_c) in addition to that of rumen outflow (k_p). Both facts lead to overestimations of the by-pass protein content (Michalet-Doreau and Ould-Bah 1989; González *et al.* 2006; Arroyo and González 2011). The combined error is especially important in fibrous forages, which require long rumen residence times for particle conditioning and are extensively colonised (Rodríguez and González 2006; Arroyo and González 2011). The above errors are also associated with an overestimation of the protein intestinal digestibility. In respect to this, González *et al.* (2009) and Arroyo and González (2011) showed that microorganisms that were adhered to feed particles in the rumen disappeared almost entirely during intestinal incubation in mobile bags and that missing k_c rate also leads to an overestimation of the intestinal digestibility. The aim of this trial was to gauge the effects of not considering the k_c rate and not correcting the rumen microbial contamination on the accuracy of *in situ* estimates of ruminal and intestinal digestion of two commonly used grass hays in ruminants.

Materials and methods

Tested forages, animals and feeding

Three Manchega breed wethers were used to estimate the *in situ* by-pass (BP) and its intestinal effective digestibility (IED) of DM, organic matter (OM) and crude protein (CP; N × 6.25) of two grass hays from oat (OH; *Avena sativa*) and ryegrass (RGH;

Lolium perenne). The BP of amino acids (AA) were also determined. These studies were performed considering two different particle transit models (either using k_p rate only or using both k_c and k_p rates) and either correcting or not the microbial contamination originating from the rumen. Wethers (59 ± 4.36 kg) were fitted with rumen and 'T'-type duodenal cannulae and housed in individual pens. For 10 days before the beginning of the experimental period, wethers were fed a 2 : 1 hay to concentrate diet, offered at 40 g DM per kg of $BW^{0.75}$ in six equal meals (every 4 h starting at 0900 hours). The diet consisted of the tested OH and a commercial granulated concentrate with 149 g of CP and 135 g of neutral detergent fibre (NDF) per kg DM. This diet contained 136 g of CP and 402 g of NDF per kg of DM. Wethers were handled according to the animal care principles as published in the Spanish Royal Decree 1201/2005 (BOE 2005). Tested hays were ground to pass a 2-mm screen for *in situ* studies and through 1-mm screen for chemical composition analysis (Table 1), including AA contents (Table 2).

Digestive transit

Transit of forage particles through the stomachs was determined by pulse dosing to each animal, before the first morning meal (i.e. at 0900 hours), 40 g of europium (Eu)-labelled OH, which was normally consumed in 20 min. The hay had previously been washed with a commercial detergent (based on sodium lauryl sulfate) in an automatic washing machine to eliminate its soluble components (Udén *et al.* 1980) and labelled by immersion in $EuCl_3$ solutions as described by González *et al.* (1998), at a dose of 10 mg Eu/g of feed. A total of 21 samples were obtained from the duodenal cannula, the first sample before supplying the marker and the remainder between 1 and 96 h afterwards. These samples were oven-dried at 60°C until humidity elimination, milled (through a 1-mm screen), and analysed for Eu. The pattern of Eu concentrations in duodenal digesta over time was described for each animal with the model of Dhanoa *et al.* (1985). In agreement with Ellis *et al.* (1979) and González *et al.* (2006), primary and secondary rate constants of this model were assumed as the rates of evacuation of particles out the rumen (k_p) and of comminution and mixing of particles (k_c) in this compartment, respectively.

In situ *studies*

Nylon bags (110 by 70 mm i.d.) with a pore size of 46 µm (Saatilon, SAATI Serigrafia Iberica SA, Almazora, Castellón, Spain) were filled with ~3 g (air-dry basis) of the hay samples. Bags were incubated in the rumen for periods of 3, 6, 12, 24, 48, 72 and 96 h. Two incubation series were performed for each feed and animal. All bags were simultaneously placed in the rumen in each series, just before the wethers were offered their first meal in the morning (i.e. at 0900 hours). Bags were washed with tap water and stored at −20°C after collected from the rumen. After thawing, bags were washed three times for 5 min in a turbine washing machine. Same procedure was applied to two series of two bags to obtain the water insoluble fraction (0-h value). Afterwards, bags were stored at −20°C once again, freeze-dried and immediately weighed to establish the degradation kinetic of DM (as proportion) in each wether in accordance with the exponential model of Ørskov and McDonald (1979):

$$d = a + b(1 - e^{-kdt}) \quad (1)$$

In this model, the constants a and b represent, respectively, the soluble fraction and the non-soluble but degradable component, which disappears at a constant fractional rate, k_d, per unit time. The undegradable fraction (r) was estimated as $1-(a+b)$. Values of these parameters are shown in Table 1. The BP of DM (DMBP) was determined in accordance with Ørskov and McDonald (1979) when only k_p was used [Eqn (2)] and in accordance with Arroyo and González (2011) [Eqn (3)] when k_c and k_p were used jointly.

$$\mathrm{DMBP} = r + b[k_p/(k_d + k_p)] \quad (2)$$

Table 1. Chemical composition and degradation kinetics of DM of tested hays

Nutrient	Oat hay	Ryegrass hay
Dry matter (g/kg)	904	916
Organic matter (g/kg DM)	889	900
Crude protein (g/kg DM)	129	115
Total analysed amino acids (g/kg DM)	68.2	73.3
Neutral detergent fibre (g/kg DM)	536	590
Acid detergent fibre (g/kg DM)	301	310
Acid detergent lignin (g/kg DM)	48.8	36.5
NDIN[A]	18.8	26.0
ADIN[A]	5.08	5.99
^{15}N abundance[B] (atoms %)	0.37071	0.37377
Dry matter degradation kinetics[C]		
a (%)	33.7 (0.29)	24.4 (0.89)
b (%)	40.0 (1.16)	60.1 (1.48)
r (%)	26.3 (1.44)	15.5 (1.63)
k_d (%/h)	5.03 (0.34)	3.84 (0.99)

[A]Neutral (NDIN) and acid (ADIN) detergent insoluble-N (% over total N).
[B]In the insoluble feed fraction.
[C]a, b and r represent soluble, non-soluble degradable and undegradable fractions, k_d fractional degradation rate of fraction b. Values in brackets are s.d.

Table 2. Amino acid proportions (g per 100 g of analysed amino acids) of oat hay (OH), ryegrass hay (RGH) and solid-associated bacteria of the rumen content (SAB)

Amino acid	OH	RGH	SAB
Arginine	3.63	4.51	5.12
Histidine	1.79	1.66	1.91
Isoleucine	4.67	5.18	5.91
Leucine	7.88	9.23	7.76
Lysine	4.53	4.39	6.91
Methionine	1.84	2.15	1.98
Phenylalanine	5.03	6.12	7.14
Threonine	4.04	4.63	5.03
Valine	6.76	6.92	5.24
Alanine	8.39	7.60	5.65
Aspartic acid	11.5	10.7	12.3
Cysteine	1.29	1.52	2.12
Glutamic acid	12.9	12.0	12.9
Glycine	5.67	7.11	5.87
Proline	14.1	9.35	2.90
Sererine	3.87	4.56	5.65
Tyrosine	2.15	2.42	5.63

$$\mathrm{DMBP} = r + b(k_c k_p)/[(k_d + k_p)(k_d + k_c)] \quad (3)$$

For each incubation time, the ruminal residues of the two incubation series for each animal were pooled in equal quantities. Then, these residues were mixed to generate composite samples (CS) representative of the DMBP that depended on whether only k_p rate or both k_p and k_c rates were considered. For this purpose, the residues for 0, 3, 6, 12, 24, 48, 72 and 96 h were considered representative for the intervals of 0–1.5, 1.5–4.5, 4.5–9, 9–18, 18–36, 36–60, 60–84 and 84–108 h, respectively. The proportions in which the different residues had to be mixed were calculated according to the rumen outflow of feed DM (Ø) in these intervals. This flow was established with function (4) (González *et al.* 1999) when only k_p was considered and with function (5) (Arroyo and González 2011) when both k_c and k_p were used:

$$\text{Ø}_{until\ t} = r(1 - e^{-k_p t}) + \frac{bk_p}{k_d + k_p}(1 - e^{-(k_d + k_p)t}) \quad (4)$$

$$\text{Ø}_{until\ t} = r\left(1 - \frac{k_c e^{-k_p t} - k_p e^{-k_p t}}{k_c - k_p}\right) + b\frac{k_p k_c}{(k_d + k_p)(k_d + k_c)} \left(1 - \frac{(k_d + k_c)e^{-(k_d + k_p)t} - (k_d + k_p)e^{-(k_d + k_c)t}}{k_c - k_p}\right) \quad (5)$$

The CS were analysed for DM, OM and CP to determine non-corrected and corrected values of the respective BP fractions (DMBP, OMBP and CPBP) and their IED (DMIED, OMIED and CPIED). The residues obtained using k_p and k_c jointly were also analysed for AA to determine their BP fractions. The BP of OM, CP and AA were determined from the concentrations of the tested fraction in the CS (Y) and in the whole feed samples (X) and the DMBP value, as follows:

$$\mathrm{BP} = \mathrm{Y} \times \mathrm{DMBP}/\mathrm{X} \quad (6)$$

The contamination of the CS was determined by labelling ruminal microorganisms with ^{15}N by a continuous intra-rumen infusion of ammonium sulfate (25 mg N^{15} per day, 98 atoms %), beginning 5 days before the start of the incubation to its end. At this moment, and before suppressing the infusion, representative samples of rumen content were obtained to isolate solid-associated bacteria (SAB) as described by Rodríguez *et al.* (2000). The isolated samples were lyophilised and analysed for DM, OM, CP, ^{15}N/N and AA. All these values are reported in the study of González *et al.* (2012); nonetheless, the AA profile is shown again in Table 2 to facilitate its comparison with the profiles of the tested hays.

After a 10-day resting period to eliminate the ^{15}N enrichment in the digesta, the IED of DM, OM and CP was determined. For this purpose eight subsamples (~200 mg) from the CS of each wether were weighed into mobile nylon bags with an approximately round shape (diameter ≈3 cm). These bags were introduced through the duodenal cannula and recovered in the faeces. Bags were inserted randomly at a rate of one bag every 15 min (eight bags per sheep a day). Subsequently, the bags were conditioned, stored and washed as described above. Then, they were dried at 70°C for 48 h and weighed. The DMIED was calculated as the disappearance of DM from the bag during the intestinal incubation. Finally, the undigested residues obtained in each wether for each transit model were pooled before OM, CP and ^{15}N/N analysis; analyses of AA were not performed because the N content in these residues was not high enough to ensure reliability. The IED of each fraction was determined from the concentration of the fraction in the CS (Y) and in the intestinal incubated residues (Z), and the value of DMIED:

$$\mathrm{IED} = 1 - [\mathrm{Z} \times (1 - \mathrm{DMIED})/\mathrm{Y}] \quad (7)$$

Microbial proportions of N and DM in the CS and in the intestinal incubated samples were determined as indicated by González *et al.* (1998). Microbial proportions of OM were established as microbial DM × OM in SAB/OM in the tested residue. The microbial contribution of a specific AA to these samples was determined as the microbial DM content × AA concentration in SAB expressed on DM.

Chemical analyses

Tested forages and incubated residues were analysed for DM (procedure 934.01; AOAC 2000), ash (procedure 967.05; AOAC 2000), and CP (N×6.25; procedure 968.06; AOAC 2000). Forage samples were also analysed for NDF (Van Soest *et al.* 1991), acid detergent fibre and acid detergent lignin (Robertson and Van Soest 1981). The insoluble nitrogen in neutral detergent and in acid detergent solutions was determined by N analysis of the respective residues. Samples of duodenal content collected for transit studies were analysed for Eu by plasma spectrometry. Nitrogen isotopic proportions were analysed by mass spectrometry (VG Prism II, IRMS linked in series to a Dumas-style N analyser EA 1108 Carlo Erba, Milan, Italy). Amino acids were determined after acid hydrolysis using α-aminobutyric acid as internal standard. Therefore, tryptophan was not measured in this study. This hydrolysis was preceded by an oxidation with performic acid to obtain methionine and cysteine values. These analyses were performed by using a Waters (Milford, MA, USA) high performance liquid chromatography system, after derivatising AA with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate.

Statistical methods

The kinetics associated to the models used were fitted using non-linear regression models. The effects of the transit model (t; k_p v. k_c, k_p) on the microbial contamination of the CS samples as well as the effects of this contamination on results of rumen-undegraded AA were studied in each forage by variance analysis by taking into account this primary factor as well as the wethers (w) in the model ($y_{ij} = \mu + t_i + w_j + \varepsilon_{ij}$). Results of BP, IED and total digested fractions of DM, OM and CP were studied by variance analyses with a split-plot arrangement of treatments ($y_{ijk} = \mu + t_i + c_j + w_k + t_i \times c_k + t_i \times w_j + \varepsilon_{ijk}$). In this design, the transit model used was the whole-plot, which was tested against the transit model × wether interaction as error term. Microbial contamination (c) and its interaction with the transit model were the subplot treatments. Differences between the AA profiles of the rumen-undegraded protein compared with that of the intact feed were analysed by performing a t-test of the differences. All statistical analyses were performed using the SAS software, version 8.0 (SAS 1999).

Results

The k_c and k_p rates of OH particles used to make up the CS of both hays were (mean ± s.d.) 22.4 ± 0.57%/h and 4.25 ± 0.421%/h, respectively. The weight of the different incubated residues in the CS is presented in Fig. 1. In both hays, the residues of the first incubation times (0, 3 and 6 h) were used at a lower proportion when k_c was considered, whereas the opposite effect was shown for the remainder incubation times. A greater ($P < 0.03$) microbial contamination on the DM, OM and CP of the CS was found for the OH when k_c was used in addition to k_p (Table 3). However, as regards the RGH, only a tendency for CP ($P = 0.088$) was observed.

Both the use of k_c and the microbial contamination correction led in both hays to decreases of BP and IED in all tested fractions (Table 4), although IED reductions due to the use of k_c did not reach statistical significance in OH. In RGH, the microbial correction led to opposite variations of digestion in rumen and intestine and, therefore, the DM or OM digested in the total tract were not affected. In CP this compensation was only partial and corrected values of CP digested showed a tendency to be higher ($P = 0.078$). Values of total digestion obtained for DM and OM using k_c showed a tendency to be higher ($P = 0.068$ and $P = 0.054$, respectively), whereas no differences were revealed for CP. The compensation between ruminal and intestinal digestion due to the microbial contamination correction was always partial in OH, with higher corrected values of total digested DM ($P = 0.055$), OM ($P = 0.057$) and CP ($P < 0.003$). The use of k_c also led to higher values of total digestion, but this effect was not demonstrated for OM. No interactions between the used transit model and the microbial correction were detected for any of the parameters of Table 4.

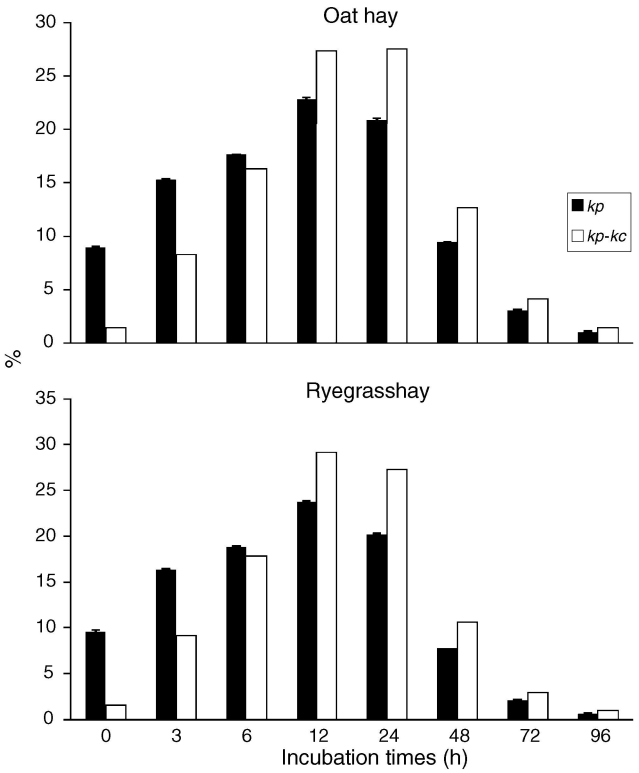

Fig. 1. Effect of the considered transit model on the composition of the representative ruminal-undegraded samples of oat and ryegrass hays. Bars are s.e.m. Significance: oat hay: $P < 0.01$: 12, 24, 72 and 96 h; $P < 0.001$: 0, 3, 6 and 48 h. Ryegrass hay: $P < 0.05$: 6 and 96 h; $P < 0.01$: 72 h; $P < 0.001$: 0, 3, 12, 24 and 48 h.

The lack of correction for the microbial contamination overestimated the BP fraction of all analysed AA, although in OH this effect was only a tendency for cysteine and proline (Table 5). These overestimations were very variable among AA with minimum and maximum for proline and tyrosine, respectively. The microbial-corrected BP of AA also showed a large variation: from 8.51% (tyrosine) to 40.0% (cysteine) in RGH and from 4.57% (tyrosine) to 28.8% (methionine) in OH. However, only cysteine and methionine showed a significant lower degradation than total analysed amino acids (TAA) in both hays as well as histidine in OH. The contrary effect was shown for tyrosine (a tendency in OH).

Ruminal degradation implied similar changes in both hays in the profile of essential AA (plus cysteine) of the undegraded protein (Fig. 2). However, in RGH variations only reached statistical significance level for leucine and valine, whose content increased in the by-pass protein as compared with the feed protein. In OH, the same increasing effect was detected for these same AA, for histidine and, as tendency, for methionine ($P = 0.063$) and arginine ($P = 0.082$). Lastly, a reduction ($P = 0.05$) was found for phenylalanine in OH after ruminal degradation.

Discussion

The process of density increase, comminution and mixing of particles required to escape from the rumen trapping mechanisms is represented by k_c and, in the case of grasses, takes a long time. As a consequence, this process always accounts for an important proportion of total rumen retention time. The proportion observed in OH (16.0%) is somewhat lower than those of 18.1 and 21.5% recorded for lucerne hay (González *et al.* 2006) and Italian RGH silage (González *et al.* 2007) in similar mixed diets. Therefore, k_c should be considered in addition to k_p in order to improve the accuracy of *in situ* estimates. This issue was raised by the ARC (1984), but this approach has been rarely put into practice, because *in situ* methods have usually been applied in a

Table 3. Effect of using the particle comminution rate (k_c) in addition to the rate of outflow from the rumen (k_p) on the microbial contamination (%) of rumen-undegraded hay samples

Hay	DM				OM				CP			
	k_p	k_c, k_p	s.e.	P	k_p	k_c, k_p	s.e.	P	k_p	k_c, k_p	s.e.	P
Oat	5.98	7.34	0.132	0.019	5.32	6.41	0.091	0.014	53.1	66.1	1.50	0.026
Ryegrass	4.26	6.71	0.634	0.111	3.76	5.87	0.595	0.124	27.1	39.1	2.68	0.088

Table 4. Effect of using particle comminution rate (k_c) in addition to the rate of outflow from the rumen (k_p) and that of correcting the microbial contamination on *in situ* estimates (%) of DM, organic matter (OM) and crude protein (CP) digestion

NC, not corrected; C, corrected; DMBP, OMBP and CPBP: by-pass of DM, OM and CP, respectively; DMIED, OMIED and CPIED: intestinal effective digestibility of the by-pass of DM, OM and CP, respectively

	k_p		k_c, k_p		Transit model		Microbial correction	
	NC	C	NC	C	s.e.	*P*	s.e.	*P*
				Oat hay				
DMBP	44.7	42.0	41.3	38.3	0.08	0.001	0.35	0.005
DMIED	6.85	2.24	5.61	1.12	0.37	0.155	0.63	0.007
Digested DM	58.4	59.0	61.0	62.2	0.21	0.010	0.22	0.055
OMBP	46.0	43.6	43.0	40.2	0.35	0.024	0.34	0.006
OMIED	9.06	4.72	6.46	1.86	1.230	0.258	0.640	0.008
Digested OM	58.2	58.5	59.8	60.5	0.77	0.233	0.20	0.057
CPBP	18.0	8.39	16.5	5.50	0.200	0.017	1.251	0.004
CPIED	31.4	3.90	40.9	2.26	2.560	0.393	3.664	0.003
Digested CP	87.8	92.1	90.3	94.6	0.30	0.027	0.911	0.029
				Ryegrass hay				
DMBP	46.8	45.4	42.9	40.0	0.35	0.011	0.33	0.012
DMIED	10.6	6.97	8.46	2.79	0.40	0.031	0.55	0.004
Digested DM	58.2	57.8	60.8	61.1	0.57	0.068	0.22	0.969
OMBP	48.7	46.9	44.1	41.6	0.37	0.011	0.18	0.001
OMIED	11.2	7.58	9.07	3.47	0.898	0.018	0.545	0.004
Digested OM	56.8	56.8	59.9	60.0	0.55	0.054	0.03	0.664
CPBP	33.0	24.0	30.4	18.9	0.31	0.009	0.73	<0.001
CPIED	51.6	34.9	47.3	18.7	1.36	0.033	3.94	0.015
Digested CP	84.1	84.4	84.1	85.5	0.38	0.389	0.27	0.078

Table 5. Effects of microbial contamination on the ruminally undegraded fraction (%) of individual and total analysed (TAA) amino acids of tested hays

NC, not corrected; C, corrected; †, $P < 0.1$; *, $P < 0.05$

	Oat hay				Ryegrass hay			
	NC	C	s.e.	*P*	NC	C	s.e.	*P*
TAA	28.3	10.2	2.97	0.049	39.4	23.6	1.95	0.029
Arginine	37.5	12.2	3.58	0.038	41.1	23.2	1.83	0.020
Histidine	39.1	19.5*	3.11	0.047	49.7	31.3	2.18	0.027
Isoleucine	33.9	10.8	3.76	0.049	42.8	24.6	2.23	0.029
Leucine	32.2	14.2	2.98	0.050	41.4	28.0	1.67	0.030
Lysine	37.4	10.6	4.16	0.045	47.0	21.8	3.59	0.038
Methionine	48.4	28.8*	2.97	0.043	48.5	33.9*	1.64	0.024
Phenylalanine	30.8	8.05	2.40	0.022	38.1	19.4	2.67	0.038
Threonine	34.1	12.1	2.52	0.025	42.0	25.0	1.48	0.015
Valine	27.5	13.4	2.19	0.045	39.8	27.8	1.39	0.026
Alanine	23.8	11.7	1.70	0.037	38.0	26.3	1.18	0.020
Aspartic acid	27.5	8.93	2.83	0.044	39.5	20.9	2.80	0.042
Cysteine	33.4	17.9*	3.09	0.071	51.4	40.0*	1.81	0.047
Glutamic acid	27.1	8.88	2.96	0.049	41.6	24.4	2.08	0.028
Glycine	33.9	15.1	2.86	0.043	36.2	23.1	1.50	0.025
Proline	9.78	5.96	0.73	0.066	21.7	16.7	0.75	0.042
Serine	36.5	11.0	3.51	0.036	43.5	23.8	2.34	0.027
Tyrosine	43.5	4.57†	2.00	0.005	45.7	8.51*	5.00	0.034
s.e.m.[A]	–	2.07	–	–	–	3.38	–	–
l.s.d.[B]	–	5.95	–	–	–	9.73	–	–

[A]Among microbial-corrected values of amino acids.

[B]Significant differences between a given amino acid and TAA.

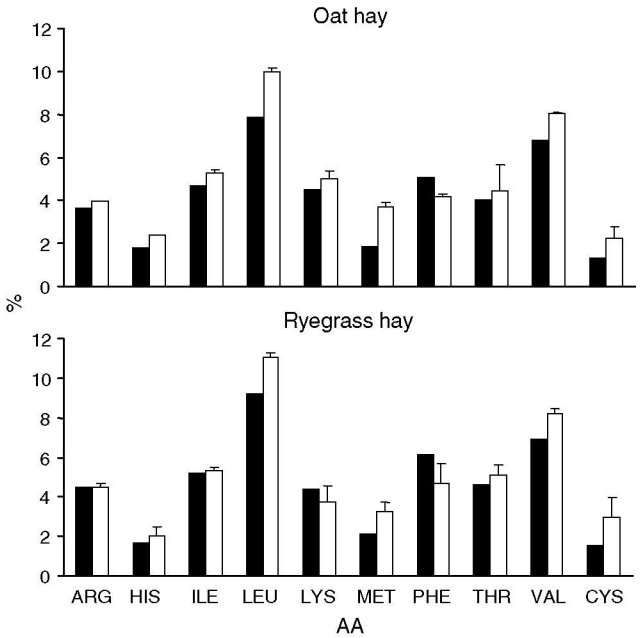

Fig. 2. Profiles of essential amino acids (plus cysteine) in feed (■) and by-pass (□) proteins of oat and ryegrass hays. Results were corrected for the microbial contamination and estimated considering the particle comminution and outflow rates. Bars are s.e.m. Effects: oat hay: $P < 0.1$: arginine, methionine, phenylalanine; $P < 0.05$: leucine, valine; $P < 0.01$: histidine. Ryegrass hay: $P < 0.05$: leucine, valine.

simplistic way. Thus, in many *in situ* studies even k_p values are assumed. Present results support the indications of González *et al.* (2006) and Arroyo and González (2011) who stressed that omitting k_c implies: (i) a large overvaluation of BP estimates in most feeds, (ii) an undervaluation of the microbial contamination of the feed BP, which enlarges the previous error, and (iii) an overvaluation of the IED of the feed BP.

The increased microbial contamination observed when using k_c is due to the exponential rise of microbial colonisation with rumen residence time (Varvikko 1986; González *et al.* 1998, 2006; Rodríguez and González 2006) and of greater inclusions of long-time incubated residues in CS (Fig. 1). Our results support those of Rodríguez and González (2006) by showing that microbial contamination is determined by chemical composition and degradative behaviour of the feed. Thus, despite the fact that OH had less fibre and more lignin than RGH, OH ended up more contaminated because of its faster degradation and its higher undegradable fraction (Table 1) favouring microorganism accumulation. In addition, our results show that the increase in contamination resulting from the use of k_c varies with the feed: 22.7 and 57.5% for DM and 24.5 and 44.3% for CP in OH and RGH, respectively, although the same transit rates (derived from OH particles) were used for both hays.

When considered independently, the non-use of k_c or the lack of microbial contamination correction resulted in moderate overestimations of DMBP and OMBP in both hays but when considered together, these errors were far from being negligible (i.e. 16.7 and 17.0% for OM in OH and RGH, respectively). As regards CP, the overestimation of CPBP with a non-microbial-corrected k_p-based model was extremely high (233 and 76.2%, in OH and RGH, respectively). Arroyo and González (2011) showed an overvaluation even higher (310%) for an Italian RGH hay, whereas González *et al.* (2006) reported a lower value (70.2%) for a lucerne hay. Since most adherent microorganisms disappear during intestinal incubation, the non-correction for microbial contamination in the rumen also leads to overestimate IED, as previously shown in an array of feeds (Arroyo *et al.* 2009; González *et al.* 2009; Arroyo and González 2011). The magnitude of this error depends on the contamination attained and on the difference between the intestinal disappearance of SAB (near 100%) and that of feed. The longer rumen residence time of particles when k_c is considered entails more extended rumen degradative actions and so, is associated with an increase in the proportion of undigestible compounds in the CS. The latter justifies the IED decreases recorded for all nutrients in RGH and numerically in OH. Values of intestinal digestibility assumed for grass hays by the PDI (70%; Vérité *et al.* 1987) and NRC systems (65%; NRC 2001) are considerably higher than results of CPIED obtained when omitting k_c and the microbial correction, which in turn, overvalue true IED estimates very largely (1289 and 176% in OH and RGH, respectively).

Effects of microbial contamination on estimates of ruminal digestion were offset by those occurred in the intestine, due to the nearly total disappearance of adherent bacteria during intestinal incubation in both hays. However, the digestion site is of utmost importance in protein and AA evaluation. Thus, microbial uncorrected k_p-based estimates undervalue the OM digested in the rumen and, therefore, the microbial CP synthesis by 9.70 and 12.2% in OH and RGH, respectively. In the same way, they overvalue the undegraded CP digested in the intestine: 5.65 v. 0.12% of total feed CP in OH and 17.2 v. 3.53% in RGH. Consequently, values usually used in forages are based on unacceptable overestimations of intestinal digested CP (González *et al.* 2006; Arroyo and González 2011). Assuming an efficiency in microbial CP synthesis of 145 g/kg of rumen fermented OM with 64% of digestible true protein (Vérité *et al.* 1987), the overestimations of intestinal digestible protein supply (microbial plus feed protein) committed by missing both k_c and contamination correction were 4.78% (51.9 v. 49.6 g/kg of feed DM) in OH and 16.8% (62.9 v. 53.8 g/kg of feed DM) in RGH.

Concentrations and profile of AA in the tested hays agree with different feed tables (Degussa 1996; Centraal Veevoederbureau 2002), except for the high proline content, especially as concerns OH. This may be due to a large deamination of arginine to proline by vegetable enzymes during the sun-curing period (McDonald *et al.* 1991).

The large contamination with microbial AA implies unacceptable errors for the estimates of the rumen-undegraded fractions in both hays. These errors were very variable among AA as they are affected by the same factors discussed for CP as well as by the difference between SAB and feeds in the concentration of each AA. Thus, minimum and maximum errors were recorded respectively for those AA with the extreme deviations between SAB and tested hays: 20–30% for tyrosine and 230–260% for proline. For a given feed, differences in AA degradability should be linked to differences among feed proteins in both AA composition and degradability (González *et al.* 2001). In this

scope, AA included in insoluble proteins (which are rich in non-polar and hydrophobic AA) are expected to be degraded to a lesser extent than those of soluble proteins. Branched-chain AA are among the most hydrophobic AA and in many studies they have been shown to be the most resistant to degradation, as reviewed by González *et al.* (2009). Liu (1999) indicated that their hydrophobicities hinder the accessibility of microbial enzymes. The increased proportions of leucine and valine in the BP protein of both hays are consistent with the foregoing observations. A low degradation of branched-chain AA has also been observed in green RGH and barley straw (Varvikko 1986) and in lucerne meal (Crooker *et al.* 1987). The hydrophobicity of sulfur-containing AA also accounted for their resistance to degradation. Cysteine is mainly present in proteins as cystine, resulting from the di-sulfur bond a strong hydrophobic character as well as a high resistance to degradation (Mahadevan *et al.* 1980). Moreover, di-sulfur bonds may contribute to maintaining the condensed structure of peptides and proteins slowing down their degradation. Accordingly, the high concentration of cysteine in RGH may be associated with its high protein degradation resistance as compared with OH. Present results of branched-chain AA, sulfur-containing AA and phenylalanine agree with the changes observed by González *et al.* (2010) in the microbial-corrected and k_c-k_p-based BP of green Italian RGH. Based on the BP of AA and on the low CPIED recorded, the contribution to the AA supply seems to be scarce, especially in OH.

Conclusions

The microbial contamination occurring in the rumen during grass incubation was revealed to be higher with a more physiological transit model that considered more accurately the rumen residence time by including the k_c rate. Thus, *in situ* results missing k_c rate and/or microbial contamination correction overestimate the by-pass fractions and their intestinal digestibility, especially that of CP. The digestible by-pass protein is low in grass hays in which the protein value is mainly determined by the microbial synthesis derived from its ruminal fermentation. Errors in the determination of rumen-undegraded AA due to the microbial contamination are very large. As a consequence, the correction becomes imperative.

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